lymph node enlargement that followed intradermal vaccination was distinctly less with cell wall vaccines than with BCG. Characteristic long-lasting histologic foci were produced by cell walls and by BCG when administered intravenously.

We are especially indebted to Miss Martha A. Redus for the readings of lymph node sizes.

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Estimation of Plasma Androgenic and Progestational Steroids in the Laying Hen (32730)

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Precise quantification of sex steroids in the chicken has been accomplished only for estrogens. Estrogens have been demonstrated in ovarian extracts (1), urine (2), and droppings (3) of the laying hen. Measurements of progesterone have been less convincing. Fraps *et al.* (4), reported the qualitative presence of progestational activity in the peripheral blood of the hen by bioassay. Layne *et al.* (5), using paper chromatography techniques, failed to find progesterone in chicken blood although they did identify this steroid in extracts of ovarian tissue. Lytle and Lorenz (6) have reported the presence of a material in ovarian venous blood that had chromatographic mobility similar to a progesterone standard. Metabolites of progesterone have not been measured. Androgens are also thought to be secreted by the hen ovary (7), but have not been quantified.

We have recently been engaged in studies to elucidate the mechanism of the induction of synthesis of a specific oviduct protein, avidin, in estrogen treated chicks by progestational and some androgenic steroids (8). Therefore, it became pertinent to attempt preliminary identification of these steroids in plasma of the mature hen using the methods presently available in our laboratory.

Materials and Methods. Nine white leghorn laying hens were anesthetized with nembutal, heparinized, and retrograde catheterization of the vena cava was accomplished by entering the left femoral vein and placing the tip of the catheter at the portion of the vena cava which drains the left ovary. This method of sampling would necessarily allow the ovarian venous effluent to be diluted by prehepatic venous blood. Plasma samples from all hens were pooled (240 ml). No attempt was made to segregate the hens according to clutch length or ovulation time.

Testosterone and Δ^4-androstenedione levels were estimated on 40 ml of the plasma pool using a double isotope technique which has been used successfully in man (9). The blank of the method determined on steroid-free human plasma is 0.010 μg/100 ml for testosterone and 0.0025 μg/ml for androstenedione. These blanks have been subtracted from the reported values.

A separate 40 ml sample of plasma was analyzed for testosterone using electron-capture gas liquid chromatography. In this meth-

od, testosterone-^{3}H is added to the plasma to monitor losses. The plasma is extracted with ether and developed by thin-layer chromatography in the system, benzene:ethyl acetate, 60:40. The testosterone containing fraction is eluted, dried, and converted to the mono-heptafluorobutyrate (HFB) after the method of Clark and Wotiz (10). The HFB's are then chromatographed in a sandwich-type thin-layer system using a benzene:ethyl acetate 95:5 liquid phase. The testosterone–HFB is eluted with 2 ml of benzene and following the addition of internal standard, an aliquot is taken for counting and a duplicate aliquot for GLC. The GLC system consists of an F & M model no. 400 operating at range 10, attenuation 16, with pulsing interval at 150 μ. The 4-foot column contains 3.5% QF-1 on Diatoport S 80–100 mesh support. Carrier gas was 95% argon, 5% methane using a flow rate of approximately 120 ml/min. No purge flow was used. In this system, the standard curves for testosterone-HFB range from 0.3 to 3.0 mμg and are linear over this range. Blank values for this method range 1–3 mμg/100 ml of plasma and are measured with each set of values.

Plasma samples were extracted three times with ether after addition of 0.5 ml of 1 N NaOH per 10 ml of plasma. The extracts were combined, washed with water and dried under reduced pressure. Aliquots of this extract were analyzed for progesterone by Dr. H. J. van der Molen utilizing an electron-capture chromatography procedure (11). Similar extracts were taken up in 70% methanol, and allowed to sit at —20°C overnight to precipitate excess fat and proteins, centrifuged, and dried. The concentrated extracts were developed on thin-layer plates in a benzene: ethyl acetate (60:40) system, followed by chromatography on Whatman no. 3 paper in the Bush A1 system (ligroin:methanol:water, 100:90:10) to separate progesterone from a fraction containing 20α-hydroxy-pregn-4-en-3-one and 20β-hydroxy-pregn-4-en-3-one. At this point of purification, GLC analysis (SE-30 column with hydrogen-flame detector) of the respective fractions revealed the presence of peaks with the identical retention times to progesterone and 20β-hydroxy-pregn-4-en-3-one standards. The above fractions were then converted to their HFB's, as described above. The HFB's were dried and developed in the same thin-layer system along with authentic standards. In this system, the HFB's of 20α- and 20β-hydroxy-pregn-4-en-3-one were incompletely separated and were eluted as a single band. These derivatives were separated from each other on subsequent GLC. Testosterone-HFB was used as the internal standard for this measurement.

Results and Discussion. The plasma levels for the various steroids measured are summarized in Table I. Testosterone (0.056 μg/100 ml) and androstenedione (0.098 μg/100 ml) were shown to be present in the pooled (ovarian–vena cava) hen plasma by the double-isotope method employed. Independent analysis by electron-capture gas chromatography revealed 0.055 μg/100 ml of plasma testosterone. The close agreement of the two testosterone values suggests that both are measuring the same steroid. Figure 1 shows the final electron-capture tracing of testosterone heptafluorobutyrate present in the purified hen plasma extract. It can be seen that the extract is reasonably free of contaminating peaks at a level of 2.15 mμg testosterone.

Progesterone was demonstrated to be present in a concentration of 0.126 μg/100 ml of plasma in the pooled hen plasma. This concentration is far below the 5.0 μg/100 ml

TABLE I. Quantitative Estimation of Androgenic and Progestational Steroids in Plasma of the Laying Hen.

	Method[a] (μg/100 ml of plasma)	
Steroid	Electron-capture chromatography	Double-isotope derivative
Testosterone	0.055	0.056
Δ^4-Androstenedione		0.098
Progesterone	0.126	
20β-OH-pregn-4-en-3-one	0.35[b]	
20α-OH-pregn-4-en-3-one	0.12[b]	

[a] See text for details of methods.

[b] Estimation does not completely account for losses during the extraction and purification. Values obtained prior to correction for losses were 0.138 μg of 20β-OH-pregn-4-en-3-one and 0.05 μg of 20α-OH-pregn-4-en-3-one/100 ml of plasma.

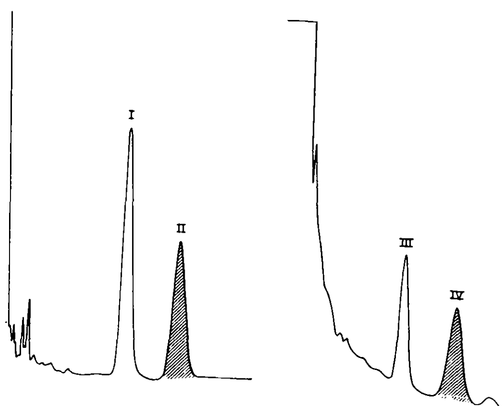

FIG. 1. Electron-capture-GLC tracings of testosterone. Left. 2 mμg of standard testosterone heptafluorobutyrate (I) and 10 mμg of 20β-OH-pregn-4-en-3-one heptafluorobutyrate, shaded (II). Right. Testosterone measured as the heptaflucrobutyrate (III) from hen plasma, and 20β-OH-pregn-4-en-3-one heptafluorobutyrate, shaded (IV) representing the internal standard.

estimation reported by Lytle and Lorenz (6) using a fluorescent-absorption technique. Significant dilution of the ovarian vein effluent by peripheral blood during our sampling technique could account for our low value. However, the presence of nonspecific fluorescing materials in the hen plasma could lead to overestimation by the fluorometric method (6).

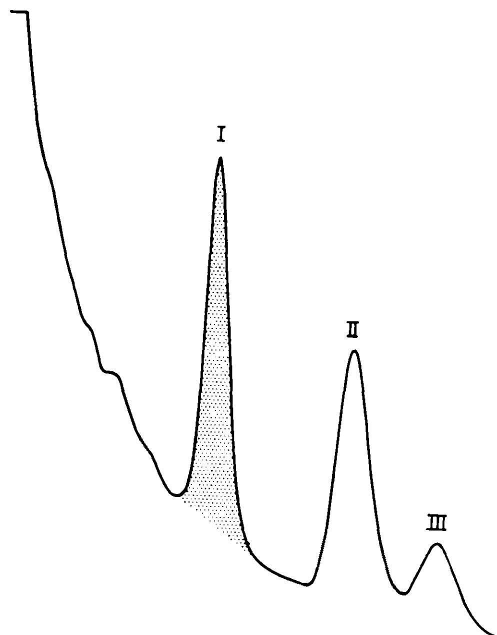

FIG. 2. Electron capture-GLC tracing of 20β-OH-pregn-4-en-3-one (II) and 20α-OH-pregn-4-en-3-one (III) measured as their heptafluorobutyrates from hen plasma. Internal standard is testosterone heptafluorobutyrate, shaded (I).

The presence of a 20α- and 20β-hydroxy-pregn-4-en-3-one in hen plasma is based on identity with reference compounds in two chromatographic systems followed by derivative formation, thin-layer, and gas liquid chromatographies. Whereas quantification of these steroids is only approximate, the predominant metabolite on repeated estimations appears to be the 20β-metabolite of progesterone (Fig. 2). This pattern is similar to that seen in the cow ovary, whereas human, mare, and sow ovaries produce predominantly the 20α-hydroxy derivative of progesterone (12).

Summary. Preliminary studies using double-isotope and electron-capture methods revealed the presence of progesterone, 20α-hydroxy-pregn-4-en-3-one, 20β-hydroxy-pregn-4-en-3-one, testosterone, and Δ^4-androstenedione in the plasma of the mature laying hen.

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